Plumbing the depths: extracellular water storage in specialized leaf structures and its functional expression in a three-domain pressure-volume relationship

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Running title: Three-domain pressure-volume relationship reflects leaf structure

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Abstract:

A three-domain pressure – volume relationship (PV curve) was studied in relation to leaf anatomical structure during dehydration in the grey mangrove, *Avicennia marina*. In domain 1, relative water content (RWC) declined 13% with 0.85 MPa decrease in leaf water potential, reflecting a decrease in extracellular water stored primarily in trichomes and petiolar cisternae. In domain 2, RWC decreased by another 12% with further reduction in leaf water potential to -5.1 MPa, the turgor loss point. Given the osmotic potential at full turgor (-4.2 MPa) and the effective modulus of elasticity (~40 MPa), domain 2 emphasized the role of cell wall elasticity in conserving cellular hydration during leaf water loss. Domain 3 was dominated by osmotic effects and characterized by plasmolysis in most tissues and cell types without cell wall collapse. Extracellular and cellular water storage could support an evaporation rate of 1 mmol $m^{-2}s^{-1}$ for up to 54 and 50 min, respectively, before turgor loss was reached. This study emphasizes the importance of leaf anatomy for the interpretation of PV curves, and identifies extracellular water storage sites that enable transient water use without substantive turgor loss when other factors, such as high soil salinity, constrain rates of water transport.

Key words: mangrove, PV curve, leaf structure, dehydration, plasmolysis, extracellular water, cisternae, trichomes, leaf water capacitance.

Introduction

Increasing vulnerability to drought is a major global concern as rise in average temperature is associated with increase in the frequency and intensity of drought in many locations (Reichstein *et al.*, 2013). Drought - induced tree mortality has been recorded worldwide in diverse ecosystems and climatic zones in the past few decades (Allen *et al.*, 2015). These reports range across a continuum of drought tolerance from species as drought sensitive as those growing in tropical rainforests (Rowland *et al.*, 2015) to the highly drought and salinity tolerant species of mangrove forests (Lovelock *et al.*, 2009). Drought-induced mortality has been linked with stem hydraulic deterioration (Anderegg *et al.*, 2012, Rowland *et al.*, 2015). Determination of stem vulnerability to drought can be difficult, especially in trees. However, Zimmermann (1983) suggested that leaves should be more vulnerable to hydraulic dysfunction than stems, as shown, for example, in sugar maple (Choat *et al.*, 2005). Indeed, leaf traits, such as the turgor loss point, can provide a powerful means of identifying species that are most vulnerable to drought (Bartlett *et al.*, 2012).

There is an urgent need to better understand the interplay of leaf water relations and leaf structure. Most recent studies have focussed on correlations between hydraulic anatomy and hydraulic conductance that inform our understanding of morphological constraints on carbon gain in relation to water loss by leaves under different environmental conditions (Sack & Scoffoni, 2013). For example, interspecific differences in vein and stomatal densities are associated with differences in leaf hydraulic conductance, and hence also in capacities for photosynthetic gas exchange under conditions of both high and low water availability (Brodribb & Holbrook, 2003). However, much less is known about the influence of other aspects of leaf anatomy on leaf water relations as measured by pressure/volume relationships.

The pressure-volume method is used to analyse the water content of a leaf in terms of water potential and its components (Scholander *et al.*, 1964, Tyree & Hammel, 1972, Cheung *et al.*, 1975). For a leaf or tissue in a pressure chamber, with the petiole protruding through a seal into air at atmospheric pressure, a plot of the pressure in the chamber vs. the volume of sap expressed from the petiole or the residual weight of the leaf (hereafter called the PV curve) yields a relationship which is usually reported as having two domains with differing behaviours. In these two-domain examples, when the

leaf water potentials are relatively high, decline in water content with increasing pressure in the chamber is dominated by quasi-elastic shrinkage of the cells as the turgor pressure is reduced. This first domain ends when turgor falls to near zero (i.e. intracellular osmotic pressure approximately equals the air pressure in the chamber). Further reduction in cell water content is determined mainly by the osmotic behaviour of the flaccid cell and is often approximated by the van't Hoff law. Thus the PV curve is information-rich, enabling the calculation and functional evaluation of parameters such as leaf capacitance for water storage (**Q**) (Tyree & Ewers, 1991), the osmotic potentials at full turgor (Ψ_{π}^{100}) and at the turgor loss point (Ψ_{π}^{0}), and an effective volumetric modulus of elasticity (**ε**), all of which are strongly associated with interspecific variation in drought tolerance (Bartlett *et al.*, 2012).

The theory underpinning interpretation of PV curves was developed with the approximation that leaves behave like osmometers (Scholander *et al.*, 1965) and that an average behaviour can be described for all cells in a leaf. However, two aspects of leaf structural complexity affect interpretation of PV curves. First, leaves are composed of a diverse range of cells specialised in different aspects of function. These cell types are structurally distinct, displaying, for example, differences in cell size and in the thickness and composition of cell walls, each of which could affect their modulus of elasticity, and hence maintenance of turgor during desiccation. In addition, even within a given cell type, variation in solute composition among adjacent hydrated cells (McCully *et al.*, 2010) could affect the osmotic potentials of individual cells at full turgor, and hence also affect the water potentials at which turgor is lost (Bartlett *et al.*, 2012). Such differences between cell types and between cells of a given type could explain the curvilinear transition between the linear elastic and osmotic domains of the PV curve (Cheung *et al.*, 1975). Indeed, Melkonian *et al.* (1982) suggested that the departure from linearity of the elastic domain of the PV curve signals the onset of turgor loss in the most vulnerable cells within a leaf; similarly, Cheung *et al.* (1975) suggested that the turgor loss point estimated from the onset of osmotic behaviour in the PV curve represents the extreme condition when all cells have lost turgor.

A second complication to interpretation of PV curves arises from the distribution of water in symplastic and apoplastic spaces within leaves. The symplastic space consists of the intracellular components of living cells bounded by the plasma membranes. The apoplastic space is more difficult to define.

Canny (1995) recognized four different appoplastic spaces: the lumina of xylem conduits and fibers, the gaseous intercellular space, and the water and Donnan free spaces of cell walls. However, more spaces may be identified as functional leaf anatomy becomes better understood. Differentiation of symplastic and apoplastic water is important in the estimation of PV parameters, such as elasticity.

In some species, PV curves with more than two domains have also been reported. The temporary accumulation of water in apoplastic spaces has given rise in some studies to a third domain at the highest levels of hydration in a PV curve. For example, in sphagnum moss, an initial domain largely corresponded to the loss of water stored in the specialised, dead, hollow hyaline cells (Hajek & Beckett, 2008). In this domain, sphagnum moss shoots lost up to 70% of water content without a measurable change in shoot water potential. Once this extracellular water was depleted, then the PV curves of drying shoots showed the two successive domains dominated by turgor reduction and osmotic relationships (Hajek & Beckett, 2008). Similar patterns were observed in other poikilohydric species of moss, lichen, liverworts, and a filmy fern (Beckett, 1997). A similar domain has also been reported in more complex homoiohydric species, such as white oak (Parker & Pallardy, 1987), *Pseudotsuga menziesii* (Mirb.) Franco (Kubiske & Abrams, 1991), and olive (Dichio *et al.*, 2003), but interpreted as an artefact of rehydration, also known as “the plateau effect” (Parker & Pallardy, 1987).

In the present study, the nature of the PV curve was related to leaf anatomy in *Avicennia marina* subsp. *australasica* (Walp.) J. Everett, a highly salt-tolerant mangrove species (Naidoo *et al.*, 2011, Nguyen *et al.*, 2015). Leaves of *A. marina* naturally endure enormous variability in hydration with leaf water potentials ranging from -0.1 MPa during rainfall events to -6.0 MPa during low tides under hot, dry weather (Constable, 2014, Walker, 2014). The leaf structure of *A. marina* is typical of C_3 species in having layers of palisade and spongy mesophyll. In addition, *A. marina* has salt secretion glands, a trichome layer on the abaxial surface, and a hypodermal water storage layer, similar to many other halophytes (Hutchings & Saenger, 1987). Thus *A. marina* is ideal for our study because it is a widely distributed species with leaves that are both highly tolerant of drought and possess complex cellular structures.

Materials and Methods

134 **Plant materials**

135 One branch was collected from each of five co-occurring trees of *A. marina* subsp. *australasica*
136 growing naturally along the Clyde River, Batemans Bay, New South Wales, Australia, and brought
137 back to the lab in a black plastic bag stored in an insulated box to prevent water loss. A hand-held
138 refractometer (A.S.T. Co. Ltd., Japan) was used to measure salinity of surface water collected at high
139 tide and soil water collected at low tide from a soil depth of 30 cm. Soil water was extracted with a
140 suction device (McKee, 1993) from three locations around each tree and measurements of these
141 three salinities were averaged to give one value per tree (n = 5 trees).

142

143 **PV curve**

144 Upon return to the lab, the cut ends of all branches were recut under a solution of 25 mM NaCl and
145 transferred to a bucket, keeping the cut ends under solution and enclosing the bucket to keep the
146 humidity high around the leaves. The branches were allowed to rehydrate overnight in the dark in a
147 cold room at 5^{o}C. The composition of this perfusion solution matched average salt concentrations
148 measured in the xylem sap of stems and leaves from *Avicennia marina* grown in seawater salinity
149 under laboratory (Ball, 1988) and natural field conditions (Stuart *et al*., 2007). Before PV curves were
150 constructed, leaves were cut from branches near the petiole – stem junctions while the petioles were
151 submerged in perfusion solution. Leaves were then transferred to a beaker, keeping the petioles
152 under solution. The beaker was covered with plastic wrap to maintain a high humidity around the
153 leaves and they were allowed to fully hydrate in the dark at room temperature for at least 30 min. The
154 leaves were then dabbed dry with tissue paper to remove excess water before measuring leaf fresh
155 mass (XP 205 Metter Toledo balance, Mettler - Toledo Ltd., Greifensee, Switzerland) and leaf water
156 potential (Pressure chamber model 1050D, PMS Instrument, Albany, USA).

157

158 Five PV curves were constructed, each made with one leaf from one tree. Leaf water potential was
159 measured at intervals corresponding to decreases in leaf fresh mass of 5-10 mg as the leaves air-
160 dried at room temperature. Time intervals between measurements ranged from 1 min at the start of
161 dehydration to 40 min for the final measurements, made when leaf water potentials reached values
162 between -5 and -6 MPa, corresponding to the minimal values Constable (2014) measured during
163 diurnal periods under field conditions in the same population of trees as in the present study. The leaf

perimeter with petiole excluded was traced on paper for leaf area measurement with a LI 3100 area meter (LICOR Inc., Lincoln, Nebraska, USA). Leaf dry mass was recorded after 48 h oven-drying to constant mass at 70°C. PV curve parameters were calculated as in Table 2 and other particulars are given in the results.

Leaf anatomy

Light microscopy

Two opposite, mature leaves were selected for anatomical analyses from the same branches that provided leaves for measurement of PV curves. One leaf in each pair was used for measurements of the tissue composition and trichome volume. Trichomes were approximated as cylinders, and trichome volumes were estimated based on height and internal lumen diameter. Transverse sections (20 - 30 μm thickness) were cut from the petiole and leaf blade with a sledge microtome (GSL 1, S. Lucchinetti, Schenkung Dapples, Zürich, Switzerland) and stained with either Toluidine blue (0.05% w/v in water) or a 50:50 mixture of Alcian Blue (1% w/v in 50% v/v alcohol) and Safranin O (1% w/v in water). The matching leaf in each pair was used to estimate trichome density, which required an inside-out epidermal preparation. The leaf epidermis was loosened by incubating the leaf in maceration solution (five parts 30% v/v hydrogen peroxide to one part glacial acetic acid) at 70°C for at least 2 h. Then the abaxial epidermis was removed with forceps, washed in water, and stained as above. Micrographs were taken at a range of magnifications using an upright microscope (DM 6000, Leica, Wetzlar, Germany) equipped with a digital camera (SPOT Flex 64MP Color FireWire 15.2, Diagnostic instruments.inc, USA) and analysed using ImageJ (Rasband, 1997 - 2014).

Cryo- Scanning Electron Microscopy (cryo-SEM)

PV curves were measured, and then leaf anatomical features, including the distribution of gas, liquid water, and cell structure, were visualized at different states of hydration using cryo – Scanning Electron Microscopy (cryo-SEM) according to McCully *et al.* (2009). One branch with four twigs similar in size was selected from each of three of the five study trees, then cut under perfusion solution (25 mM NaCl) and left for a few hours until fully hydrated. Then the four twigs, each with at least three pairs of fully expanded leaves, were cut under perfusion solution from each branch. One twig from each branch was sampled immediately upon removal from perfusion solution and the

194 remaining three twigs of each branch were then air-dried for differing periods of time before sampling
195 to capture change in leaf structure associated with key features of the previously measured PV
196 curves. At each sampling time, one leaf in each pair was used to measure leaf water potential while
197 the other leaf was frozen rapidly (less than 0.1 s) with cryo-pliers cooled with liquid N_2 (LN2) (McCully
198 *et al.*, 2010). Frozen pieces (1 - 2 x 0.5 cm) were cut from the desired region under LN2, placed in
199 cryo-vials and stored in LN2 until processed for cryo-SEM. Then, frozen leaf segments were trimmed
200 to smaller pieces (3 mm length) under LN2, planed with a cryo-microtome (Leica EM FC7, Leica,
201 Wetzlar, Germany) and visualised by cryo-SEM (Hitachi 4300SE/N, Hitachi High Technologies,
202 Japan) following McCully *et al.* (2010).
203
204 **Mucilage Analysis**
205 Sample preparation
206 Gel extruded from petioles of *A. marina* was collected and dispersed in 5% EtOH/water. The solution
207 was centrifuged to remove insoluble extraneous matter and the supernatant freeze-dried.
208
209 Polysaccharide analysis (% composition)
210 The reductions of the constituent uronic acid methyl esters and the free uronic acids were carried out
211 following the protocol of Kim and Carpita (1992) and of Pettolino *et al.* (2012). The reduced
212 polysaccharides were then hydrolysed, reduced, acetylated and subject to gas chromatography mass
213 spectrometry (GC/MS) analysis as described by Peng *et al.* (2000).
214
215 **Water uptake by trichomes**
216 Three twigs, each with a fully mature leaf pair, were harvested from three saplings of *A. marina* that
217 had been hydroponically grown (nutrient solution salinized with addition of 250 mM NaCl) from
218 propagules that originated from the same field populations used for PV curve analyses. Leaves were
219 rehydrated and then air-dried, with water potential determined at intervals during drying as described
220 above for PV curves. Following each measurement of leaf water potential, leaves were tested for
221 uptake of water by the trichome layer that covered the abaxial leaf surface. Upon removal from the
222 pressure chamber, a leaf was positioned to view the trichome layer under an epi-fluorescence
223 microscope (Zeiss Axiostar plus, Carl Zeiss, Germany). A drop of 0.1% fluorescein sodium salt

224 solution (British Drug Houses, Poole, England) was applied to the trichome surface and fluorescence
225 from the dye was observed by standard methods under blue-exciting light. A threshold for water
226 uptake by trichomes was determined by the highest leaf water potential at which dye applied to the
227 abaxial leaf surface disappeared into the trichomes. This threshold was used to estimate the onset of
228 drainage from trichomes as well-hydrated leaves were air-dried.
229
230 **Data analysis**
231 Data analyses using simple linear regression or one-way ANOVA were performed in Genstat version
232 16 (Payne, 2014). Data did not require transformation prior to analysis. Where relationships were
233 significant, differences between treatment means were tested *post hoc* for significance (here
234 considered $P \leq 0.05$) using Fisher's Least Significant Difference test. Unless otherwise stated, all
235 results given in the text are mean values ± standard error (se), n = 5.
236
237 **Results**
238
239 **PV curve analysis**
240 All measurements were made on leaves collected from mature trees that grew naturally where soil
241 water salinity at a depth of 30 cm averaged 49 ± 0.5 ppt (parts per thousand by weight) and surface
242 water at high tide averaged 35 ppt. For reference, standard seawater has a salinity of 35 ppt, and an
243 osmotic potential of -2.4 MPa. Thus the roots were exposed to soil and surface water with an osmotic
244 potential ranging from -3.4 to -2.4 MPa, respectively. However, surface water salinity can become
245 higher during low tide when drying concentrates salt at the soil surface.
246
247 Characteristics of the leaves used for PV curve measurements are summarised in Table 3. Fully
248 expanded, sun leaves of *A. marina* had an average area of 14.8 ± 1.1 cm^2 and contained 0.66 ± 0.05
249 g of water when fully hydrated. Relative water content (RWC) was measured on these leaves as a
250 function of leaf water potential (Ψ_{leaf}) during air-drying. Variation in RWC with Ψ_{leaf} followed a
251 complicated relationship with three domains as shown in Fig. 1a.
252

253 Water relations parameters of *A. marina* leaves were calculated from plots relating water content to
254 water potential (commonly called PV curves) and are summarised in Table 4. Domain 1 of the curve
255 was characterised by rapid decline in RWC produced by relatively small application of pressure in the
256 chamber. On average, RWC declined 13 ± 1% in this domain, for which there was less than 1 MPa
257 decrease in the inferred leaf water potential. Water potential at the transition from the first to the
258 second domain (Ψ_x) averaged -0.85 ± 0.06 MPa (point ***B***, Fig. 1a). Domain 2 covered the largest
259 variation in Ψ_{leaf}: a decrease from -0.85 to - 5.1 MPa, five times larger than that of domain 1. Over the
260 second domain, RWC decreased by an average of 12 ± 3%, almost as much as that of domain 1.
261 Finally, domain 3 was characterized by more rapid rate of leaf water loss for a given decrease in leaf
262 water potential than the previous domain, domain 2. The transition between domains 2 and 3 (point ***C***,
263 Fig.1a) averaged -5.1 ± 0.1 MPa. Given the salinities of pore and surface water, Ψ_{leaf} at this transition
264 was inferred to be the leaf water potential at turgor loss point (Ψ_π^0), which was supported by
265 calculations from the fitted straight line (dashes and points) in Fig. 1b as shown below.
266
267 The data set used in Fig. 1a was replotted in Fig. 1b with the reciprocal of Ψ_{leaf}, ($|1/\Psi_{leaf}|$) plotted as a
268 function of relative water content deficit, calculated with total leaf water content (RWD+, %). The
269 starting point, **c**, of the linear relationship between $|1/\Psi_{leaf}|$ and RWD+ indicated the reciprocal of leaf
270 water potential at the turgor loss point ($|1/\Psi_\pi^0|$); further, the intercept $\mathbf{d_x}$ is given by the coordinates
271 RWD+ = RWD_x+, i.e. the percentage of water content decrease in domain 1, and $|1/\Psi_{leaf}| = |1/\Psi_\pi^{100}|$,
272 i.e. the reciprocal of the osmotic potential at full turgor. According to these calculations, leaf water
273 potential at the turgor loss point and osmotic potential at full turgor averaged -5.1 ± 0.1 MPa and -4.2
274 ± 0.1 MPa, respectively, in *A. marina* leaves. These calculations excluded extracellular water implied
275 in domain 1. If that water was included, then Ψ_π^{100} would be calculated from the $1/\Psi_{leaf}$ intercept, $\mathbf{d_0}$,
276 as shown in Fig. 1b and would average -3.5 ± 0.1 MPa.
277
278 The PV curves were replotted without domain 1, as suggested by Beckett (1997) and Dichio *et al.*
279 (2003) to account for effects of extracellular water (the plateau effect) on calculation of leaf water
280 relations parameters from the PV curve (Supplement S1). Calculation of Ψ_π^0 was unaffected by
281 removal of domain 1, but Ψ_π^{100} was changed because the method of Beckett (1997) and Dichio et al.
282 (2003) redefined the status of a fully hydrated leaf. Specifically, calculation of maximum leaf water

content (WC_{max}) changed from (FM_{max} - DM) to (FM_{max} - WC_{ex} - DM), where FM_{max} is the maximum leaf fresh mass, DM is the leaf dry mass, and WC_{ex} is the extracellular water content. As WC_{max} decreased, RWC increased accordingly (see Table 2 for RWC calculation). Upon the removal of domain 1, Ψ_{π}^{100} was calculated at the $1/\Psi_{leaf}$ intercept of the linear regression between inverse leaf water potential ($|1/\Psi_{leaf}|$, MPa^{-1}) and relative water deficit, calculated without extracellular water, (RWD-, %), i.e. point **d** (Supplement S1b), and averaged -4.2 ± 0.1 MPa. This value was equal to that calculated at $\mathbf{d_x}$ (-4.2 ± 0.1 MPa, P = 0.968) but significantly different from that calculated at $\mathbf{d_0}$ (-3.5 ± 0.1 MPa, P <0.001).

Calculation of water storage capacitance and bulk modulus of elasticity

The area-specific water storage capacitance (called water storage capacitance hereafter) was calculated as the mass of water lost per unit area per unit decrease in water potential (Table 2). Two values of water storage capacitance were calculated from different points on the PV curve (Table 4). Water storage capacitance of domain 1 ($\mathbf{Q_x}$) was calculated from points ***A*** to ***B*** (Fig. 1a) and the capacitance of domain 2 ($\mathbf{Q_e}$) was calculated from points ***B*** to ***C*** (Fig. 1a). $\mathbf{Q_x}$ averaged 69 ± 6 g m^{-2} MPa^{-1}, and $\mathbf{Q_e}$ averaged 13 ± 4 g m^{-2} MPa^{-1}. In other words, application of a chamber pressure of 1.0 MPa during dehydration in domain 1 would release approximately 69 g water per m^2 leaf area whereas similar application of 1.0 MPa in domain 2 would release about 13 g water per m^2 leaf area. The average storage capacitance over both domains ($\mathbf{Q_g}$, points ***A*** to ***C***, Fig. 1a) was 22 ± 4.8 g m^{-2} MPa^{-1}.

The effective bulk modulus of elasticity, $\boldsymbol{\varepsilon}$, is defined as the change in chamber pressure or water potential required for a fractional decrease in a cell's water volume ($\boldsymbol{\varepsilon} = \Delta P/(\Delta V/V)$). The compressibility of water over this range of pressures is negligible, so $\boldsymbol{\varepsilon} = \Delta P/\Delta RWC$. The values of $\boldsymbol{\varepsilon}$ thus estimated (Table 4) averaged 7 ± 1 and 37 ± 10 MPa for domains 1 ($\boldsymbol{\varepsilon_x}$) and 2 ($\boldsymbol{\varepsilon_e}$), respectively, with an average value, $\boldsymbol{\varepsilon_g}$, of 21 ± 2 MPa calculated over both domains.

310 Water storage capacitance and elasticity calculated upon the removal of domain 1 averaged 13 ± 2 g
311 $m^{-2} MPa^{-1}$ and 38 ± 4 MPa, respectively, and were not significantly different from those calculated
312 from domain 2, i.e. $\mathbf{Q_e}$ and $\boldsymbol{\varepsilon_e}$, (P = 0.383, and P = 0.841, respectively).
313
314 **Leaf structure**
315 **Petiole**
316 The basic features of petioles and leaves were characterised by bright field microscopy (Fig. 2).
317 Petioles (Fig. 2a) of *A. marina* leaves were covered with two types of multicellular trichomes.
318 Trichomes of the first type were clustered together with mucus secretion glands in a groove at the
319 petiole - stem junction. Trichomes of the second type covered the rest of the petiole and leaf abaxial
320 surfaces where the trichomes coexisted with salt secretion glands. These trichomes had a rivet-like
321 shape with a cap cell on top, one to two stalk cells in the middle, and a basal cell at the bottom. The
322 cap and stalk cells were dead with a hollow lumen. Trichomes of both types were transparent;
323 however, they appeared black if cyanobacteria were present.
324
325 The cortex was the largest tissue within the petiole and contained two distinct layers. A layer of
326 collenchyma (6 -7 cells thick) occurred beneath the epidermis, and was underlain by a layer of
327 parenchyma (15 - 16 cells thick). Substantial gas spaces occurred between parenchymal cells. The
328 gas spaces in the petiole cortex extended into the bundle sheath extension of the midvein but not to
329 the higher vein orders. The remaining space in the petiole was occupied by the two fimbrial veins and
330 the central vascular cylinder which contained a circular array of vascular bundles as commonly found
331 in eudicots. However, xylem vessels were not evenly distributed among the vascular bundles, with
332 more vessels occurring in bundles near the abaxial than adaxial sides of the petiole. Finally, the pith
333 occupied the most central space, surrounded by vascular tissue. The pith consisted mainly of tightly
334 packed parenchymal cells interspersed with small phloem bundles.
335
336 **Leaf lamina**
337 Leaves had a reticulate venation system with a prominent midvein as shown in Fig. 2b. A thick cuticle
338 covered the adaxial leaf surface except where salt secretion glands occurred at the base of scattered
339 depressions in the leaf surface. These salt secretion glands were in contact with underlying epidermal

340 and hypodermal cells. The midvein vascular structure was similar to that of the petiole. Nevertheless,
341 a ring of fibers surrounding the central vascular cylinder was more developed in the midvein than in
342 the petiole. In place of the cortex, the midvein was bounded by a bundle sheath extension (BSE) that
343 connected the vascular tissue with the upper and lower epidermis. Leaves of *A. marina* were
344 heterobaric but bundle sheath extensions only occurred at vein orders ranging from 1^{st} to 4^{th}. The
345 midvein was also linked with adjacent mesophyll and hypodermal tissues (Fig. 2c).
346
347 Four major tissues contributed most of the leaf lamina thickness (Table 3). The structure of *A. marina*
348 leaves was typical of C_3 species, with veins embedded between the palisade and spongy mesophyll
349 (Fig. 2c). Spongy mesophyll thickness was about two thirds that of palisade mesophyll. Together, they
350 accounted for 43 ± 1% of total leaf thickness. *Avicennia marina* leaves had an additional hypodermal
351 layer located below the upper epidermis, accounting for about 36 ± 1% of leaf thickness. Trichomes
352 on the abaxial leaf surface were similar in structure to the rivet-like trichomes on the petiole but
353 longer, and accounted for 19 ± 1% of total leaf thickness. Salt secretion glands occurred on both leaf
354 surfaces, but stomata were only on the abaxial surface.
355
356 **Change in leaf structures during drying**
357 Leaves were cryo-preserved during drying to fix the cell structure and the spatial distribution of gas
358 and liquid. Changes in these three components of leaf structure were assessed in relation to the three
359 domains of the PV curve using cryo-SEM. Domain 1 was characterized by the occurrence of
360 extracellular water in the gas spaces of the rivet-shaped trichomes, petiole, bundle sheath extension,
361 and leaf lamina. Extracellular water was not observed in samples taken in domain 2. Changes in cell
362 RWC could not be determined from the images because of variation in cell size and shape between
363 leaves. However, during domain 3, plasmolysis was apparent in most cells, but no cell wall collapse
364 was observed except in the trichomes.
365
366 Trichome water status changed with leaf water status. For reference, general trichome structure is
367 shown in Fig. 3a. Trichome lumina were full of liquid water when leaves were fully hydrated (Fig. 3b).
368 During domain 1, this extracellular water was replaced with gas (Fig. 3d) while the stalk cells of

369 trichomes retained their shape; however, the cap cells were collapsed (Fig. 3c, d). In domains 2 and
370 3, both cap and upper stalk cells were shrivelled and collapsed (Fig. 3e).
371
372 Additional measurements using fluorescence microscopy on fresh materials demonstrated the onset
373 of trichome drainage with dehydration (Fig. 4). When viewed under white light the cap cells of the
374 trichomes formed a continuous surface (Fig. 4a). The hollow stalk cells appeared like dark circular
375 areas centred beneath the cap cells. No structure was visible under blue light showing the absence of
376 detectable auto-fluorescence (Fig. 4b). In contrast, the presence of fluorescein was detected on the
377 leaf surface by bright green fluorescence under blue light (Fig. 4c - f). When trichomes were filled with
378 water ($\Psi_{leaf} > 0.1$ MPa), fluorescein quickly spread over the leaf surface without loss in fluorescence
379 (Fig. 4c, d and movie 1(Supplement S2)). Drainage of water-filled trichomes was detected when Ψ_{leaf}
380 declined to -0.25 ± 0.03 MPa (n = 3). Under these conditions, fluorescein drops remained where
381 applied for approximately a minute before rapidly disappearing into the trichomes (Fig. 4e, f, and
382 movie 2 (Supplement S3)). The estimated density and volume of trichomes (Table 3) indicated that
383 they could potentially hold up to 10% of total leaf water.
384
385 Petiolar gas spaces also functioned in temporary water storage of well-hydrated leaves, and,
386 therefore, were named cisternae. Due to the complex three-dimensional structure, it was not possible
387 to obtain a reliable estimate of water storage in cisternae from the micrographs. When viewed by
388 bright-field microscopy, longitudinal sections through the petiole revealed greater surface structure
389 delimiting these cisternae than expected (Fig. 5a, b). Closer inspection under cryo-SEM revealed that
390 the walls of these cisternae had a rough surface that appeared coated with a mucus-like substance
391 that formed globular structure, hereafter called droplets, with drying. Analysis of a gel extruded
392 naturally from petioles revealed a composition consistent with mucilaginous polysaccharides (Table
393 5). When leaves were well hydrated (Ψ_{leaf} = -0.1 MPa), most cisternae were full of water but some
394 contained large droplets with diameters averaging 6.8 ± 0.8 µm (n = 20) (Fig. 5c, d). Where the
395 planing knife cut through the ice of a water-filled cistern, the eutectic domains of the ice indicated
396 water of high solute content. At the end of domain 1, no cisternae were filled with water and droplet
397 diameter averaged 4.9 ± 0.3 µm (n = 20) (Fig. 5e), smaller than that observed at higher leaf water

potentials. As leaf water content decreased over domain 2, very few droplets were found and their average diameter was 2.2 ± 0.3 µm (n = 20) (Fig. 5f).

In the leaf lamina and midvein, small gas spaces between pith parenchyma and between mesophyll cells were also filled with liquid water but only when leaf water potential was less negative than -0.1 MPa (Fig. 6). Where the planing knife cut through the ice of a water-filled intercellular gas space, the general absence of eutectic domains in the ice indicated water of very low solute content, in contrast to that of the cisternae. The boundary of the lamina spaces was not specially coated like that of the cisternae. No gas space or external water was found between hypodermal cells. Due to variability in cell sizes within and between leaves, it was not possible to quantify changes in size of the hypodermal cells over domains 1 and 2.

In domain 3, a wide variety of cell types, including collenchyma and parenchyma cells in the petiole and the hypodermal, mesophyll cells, and epidermal cells in the leaf lamina, showed similar responses to decreases in leaf water potential below the turgor loss point. Gaps appeared between cell walls and the enclosed cells, as is characteristic of plasmolysis (Fig. 7).

Discussion

The results of the present study revealed a new twist in an old curve and novel insights into the interpretation of the PV curve in *Avicennia marina*. This PV curve exhibited three domains over the range of hydration in which the leaves naturally function under field conditions, even in a single day (Constable, 2014, Walker, 2014). The three domains of leaf dehydration were characterized by (1) the presence and consumption of extracellular water, (2) decline in turgor, and (3) the occurrence of plasmolysis. Each domain, as illustrated in the summary diagram (Fig. 8), and its implications for leaf function, are discussed in detail below.

Domain 1: dominated by variation in extracellular water

The domain of highest hydration was characterised by the presence of extracellular water and its disappearance as RWC decreased by 13% while leaf water potential only declined from -0.1 to -0.85

MPa (Fig. 1a, Fig. 8). At the highest hydration level, water filled extracellular spaces, including those between mesophyll cells (Fig. 6c), the hollow cores of trichomes (Fig. 3b), and the petiolar cisternae (Fig. 5c). As the leaf dried, water was replaced by gas in these distinct spaces (Figs. 3d, 5e, f, 6d) over different ranges of water potentials, implying that filling of the spaces occurred under different conditions and depended on different processes.

Liquid water was only observed in some mesophyll extracellular gas spaces when water potential was less negative than -0.1 MPa. Water storage between mesophyll cells would have a negative impact on gas exchange because diffusion of CO_2 is about 10,000 times slower in water than in air, but the extent of this storage was small and short-lived. The lack of eutectic domains in this extracellular water that had been cryo-preserved indicated that the water had very low solute concentrations. How can we account for movement of liquid water from cells to extracellular gas spaces in the absence of an osmotic gradient? The answer may be similar to processes driving refilling of embolized protoxylem vessels in well-hydrated tissues (Rolland *et al.*, 2015). Once the cells had achieved high water potentials, capillary forces due to the combination of hydrophilic walls and close contact between cells may have driven extracellular water accumulation in confined spaces. This limited filling of extracellular gas spaces in excised leaves was not an artefact as similar observations have been made on *A. marina* leaves under natural conditions when they were collected predawn following exposure to nocturnal wetting events (Constable, 2014). Such accumulation of extracellular water in these well-hydrated leaves presumably occurred through foliar absorption of atmospheric water, even though roots grew in seawater; in other words, the water potential gradient during leaf wetting events was reversed from the atmosphere to the leaves to the soil.

The presence of low solute concentrations in the extracellular water has important implications for salt tolerance of halophytes. During symplastic water uptake at the roots (Moon *et al.*, 1986), *A. marina* excludes approximately 90 - 95% of salt in soil water from entry to the transpiration stream (Ball, 1988). When grown in seawater, *A. marina* typically has 25 mM NaCl in the transpiration stream (Ball, 1988, Stuart *et al.*, 2007). In *A. marina*, water and associated salt are distributed across a leaf lamina through the vascular system to vein endings and subsequently dispersed to client cells by symplastic pathways (Fitzgerald & Allaway, 1991). This symplastic control prevents catastrophic salt

458 accumulation in apoplastic spaces and facilitates salt management within a leaf. In *A. marina*, part of
459 the salt transported in the transpiration stream is accommodated in growing cells for osmotic
460 adjustment of the vacuoles while the remainder is exported from the leaves via either the phloem or
461 epidermal salt secretion glands, thereby maintaining a favourable salt balance (Ball, 1988). Thus the
462 observation of low solute concentrations in the extracellular water (Fig 5d) is consistent with strong
463 symplastic control of both water and associated salt distribution within the leaves (Fitzgerald &
464 Allaway, 1991).
465
466 Water-filled trichomes began to empty when Ψ_{leaf} declined below -0.25 MPa (Fig. 4e, f and movie 2
467 (Supplement S3)). In contrast to intercellular spaces in the mesophyll, the trichome lumina appeared
468 to contain a mucilaginous substance. This was suggested by the similarity of the ice texture in water-
469 filled trichomes that had been cryo-preserved (Fig 1b) to that observed in cryo-preserved
470 mucilaginous secretions of root tips (McCully *et al.*, 2009). Such a hydrophilic substance in
471 combination with the highly hydrophilic walls and the small diameter of the hairs could have facilitated
472 water uptake at relatively high water potentials, as might occur with exposure to rain or dew. Indeed,
473 under natural field conditions, Constable (2014) observed trichomes to be full of water when leaves of
474 *A. marina* were wet in the morning. Trichomes seemed to serve as one of the main water storage
475 sites, which could hold up to 10% of total leaf RWC. This storage water could be released to the leaf
476 as leaf water potentials declined with dehydration if water transport into the leaf followed a symplastic
477 pathway through the living basal cells of the trichomes to the underlying cells (Fig. 3a). Similarly,
478 water absorbed by trichomes contributed to the water status of underlying leaf cells in oak leaves
479 (Fernández *et al.*, 2014). Thus, identification of trichomes as a major water storage site in *A. marina*
480 also implies that the trichomes may provide a means for rehydration via foliar water uptake.
481
482 Finally, the present study identified a novel water storage structure, here named the cisternae, which
483 occurred mainly in the petiole and extended into the bundle sheath extension of the midvein near its
484 junction with the petiole. Extracellular water occurred in cisternae until leaf water potentials declined
485 to approximately -0.8 MPa. Unlike the extracellular spaces in the mesophyll and the trichomes, the
486 cisternae were relatively large, completely bounded by the walls of cells comprising the surrounding
487 tissues (Fig. 5a,b), and appeared to contain a combination of mucilage, mostly arabinose, and a

relatively high concentration of solutes (Table 5, Fig. 5d, f). The latter must have been sufficient to attract water from the surrounding cells into the cisternae when leaf water potentials rose above -0.8 MPa. Extension of the cisternae into BSE of the midvein would enhance the reach of their influence on leaf water relations as the BSEs are known to play a role in extravascular water dispersal (Canny, 1986, Buckley *et al.*, 2011, Sommerville *et al.*, 2012, Zsogon *et al.*, 2015). Conversely, because the osmotic potential of the cisternae would vary with water content, differential drainage of the cisternae during leaf drying could contribute to the curvature linking domains 1 and 2 in the PV curve.

Domain 2: Dominated by variable turgor

The second domain was characterized by 12% loss in leaf RWC as leaf water potentials declined from -0.85 to -5.1 MPa. Decrease in cell volume, except for that in trichomes, was not obvious in our micrographs, possibly due to the small scale of total loss in RWC (i.e. 12% across domain 2, Fig. 1a), combined with high variability in cell sizes (Fig. 8). For example, a 12% change in leaf water content would be equivalent to a 4% change in each dimension of an isotropic cell.

Moreover, there were no obvious changes in gas spaces between mesophyll cells with decreasing Ψ_{leaf}, consistent with observations of desiccating sunflower leaves (Fellows & Boyer, 1978). Tissue connections in the leaf lamina were maintained as cell walls and membranes also maintained contact during shrinkage (i.e. cytorrhysis). Given the large osmotic potential at full turgor (Ψ_{π}^{100} = -4.2 MPa) and the high modulus of elasticity, most of the decline in leaf water potential with decrease in RWC in the second domain was associated with decreasing turgor pressure.

The curvature between domains 2 and 3 may reflect differential turgor loss in different cell types with the turgor loss point representing the extreme case where all cells have lost turgor (Cheung *et al.*, 1975). Some cells might lose water more rapidly than others due to their properties or their positions within the leaf. In addition, **ε** is likely to be itself a function of turgor. For example, the high and constant **ε** region could represent cell wall stretching, and the lower and variable modulus region could represent change in shape. Thus, the curvature shown linking domains 2 and 3 (Fig. 1) likely reflects the large contribution of different cell types to water relations in *A. marina.* Similar curvature was found in many studies where PV curves were constructed from repeated measurements on a

single leaf or shoot (Cheung *et al.*, 1975, Meinzer *et al.*, 1986, Parker & Pallardy, 1987). In contrast, a sharp transition between domains 1 and 2 has been observed when the PV curve is constructed from a composite of measurements made on many leaves of a species, such as those from woody tropical rainforest (Brodribb & Holbrook, 2003) or used in theoretical analyses, e.g. Suarez and Sobrado (2000). The convexity of the transition may make a useful tool for comparing hydraulic complexity in leaves grown under different conditions or between different species.

Domain 3: Dominated by osmotic behaviour of flaccid cells

Domain 3 was characterised by plasmolysis (Figs. 7, 8) as leaf water potentials declined below the turgor loss point once RWC decreased below approximately 75% (or below about 86% if the extracellular water was not included in the accounting) (Table 4). In most living cell types, connections between tissues or between cells comprising a tissue were maintained during desiccation-induced shrinkage. No collapse of cell walls was observed. However, separation between cell walls and plasma membranes developed when Ψ_{leaf} was about 1 MPa lower than Ψ_{π}^{0}. Plasmolysis in *A. marina* was also noticed in the field when Ψ_{leaf} was as low as -6 MPa, and the cells recovered following nocturnal rehydration (Constable, 2014). The fact that *A. marina* leaves could recover after reaching the turgor loss point was consistent with a study by Brodribb and Holbrook (2003) which showed that irreversible damage to PSII system only occurred in woody tropical rainforest species at Ψ_{leaf} much lower than Ψ_{π}^{0}.

Ecophysiological implications of the new PV curve to *A. marina* function

The results of the current study emphasized the importance of leaf anatomy to leaf water relations with far reaching implications for leaf function under field conditions. Previous studies have referred to the first domain as “the plateau effect” (Parker & Pallardy, 1987, Dichio *et al.*, 2003), attributed to artefactual rehydration. The presence of domain 1 due to extracellular water does not change values of PV curve parameters, including Ψ_{π}^{0}, Ψ_{π}^{100}, $\boldsymbol{\varepsilon}$, and **Q**, as long as those parameters are calculated using data from appropriate domains as shown in Fig. 1 and indicated in Table 4, consistent with recommendations to account for “the plateau effect” (Parker & Pallardy, 1987). However, dismissal of

546 domain 1 as an artefact of rehydration would lead to false assumptions about leaf functions in *A.*
547 *marina* that are inconsistent with field observations and leaf anatomy.
548
549 The PV curves revealed a high modulus of elasticity in leaves of *A. marina*, which plays a major role
550 in cell function over the whole range of water potentials naturally experienced by these leaves. At
551 maximum hydration, leaf water potentials were very high (i.e. -0.1 MPa) while osmotic potential at full
552 turgor was -4.2 MPa (Fig. 1b). The turgor pressure would therefore have been around 4.1 MPa, but
553 there was no evidence that cells burst under these conditions. Presumably, the high modulus of
554 elasticity, i.e. 37 MPa (Table 4), contributed to cell survival when the leaves were fully hydrated.
555
556 Previous studies have interpreted the role of a high **ε** on leaf function under low water potentials in
557 two ways. First, high **ε** was proposed to enhance water uptake into a transpiring leaf by increasing
558 the water potential gradient between the leaf and the soil (Bowman & Roberts, 1985). However,
559 Bartlett *et al.* (2012) argued that this proposal disregards the role of hydraulic conductance in
560 determining the water potential gradient. Indeed, studies of diurnal gas exchange in the same
561 population of *A. marina* as the present study showed that reduction in leaf hydraulic conductance
562 began when leaf water potentials declined below -3 MPa (Walker, 2014). Consequently, decline in
563 Ψ_{leaf} with decreasing turgor would not necessarily enhance water uptake into leaves as they dried.
564 Indeed, a low **ε** would allow the concentration of the cytoplasm to vary significantly over the range of
565 positive turgor, whereas a high **ε** would keep the cytoplasmic concentrations similar, and also reduce
566 the possibly deleterious effects of changes in cell geometry. Thus, we agree with the second
567 suggestion that a high modulus of elasticity coupled with a low solute potential enables leaves to
568 maintain the levels of hydration required for cellular function during drying to the turgor loss point
569 (Cheung *et al.*, 1975, Bartlett *et al.*, 2012).
570
571 The extent of water storage in domains 1 and 2 revealed by PV curve could play an important role in
572 the maintenance of diurnal photosynthetic activities in leaves of *A. marina*. Specifically, extracellular
573 water storage capacitance of domain 1 ($\mathbf{Q_x}$) was about five times greater than the intracellular water
574 storage capacitance of domain 2 ($\mathbf{Q_e}$). However, $\mathbf{Q_x}$ was functional over a range of Ψ_{leaf} approximately

five times less than that of $\mathbf{Q_e}$. Consequently, extra and intracellular water storage was about equal. In other words, storage of water in extracellular spaces doubled the amount of water available for use in gas exchange without loss in turgor. For example, area-specific evaporation rates measured for *A. marina* grown at the same location as those in the present study ranged from 1 to 2 mmol H_2O m^{-2} s^{-1} (Martin *et al.*, 2010). So a leaf with extracellular water storage capacitance of 69 g H_2O m^{-2} MPa^{-1} (Table 4) or 3800 mM H_2O m^{-2} MPa^{-1} as estimated from domain 1, when fully charged, could alone supply the water loss needed to support photosynthesis at the above evaporation rates for 3230 to 1615 s, that is for 54 to 27 min with only 0.85 MPa decrease in Ψ_{leaf}. Once extracellular water is exhausted, then the water storage capacitance of domain 2 (i.e. 13 g H_2O m^{-2} MPa^{-1} or 700 mM H_2O m^{-2} MPa^{-1}) could alone support the same rates of gas exchange for another 50 to 25 min until the turgor loss point (i.e. -5.1 MPa) is reached. Given the relative proportions of space occupied by different tissue layers in the leaf lamina (Table 3), the hypodermis alone could have buffered water loss from the mesophyll by contributing almost half of the water available for photosynthesis during dehydration over domain 2 (all in the absence of water flux from the roots). Thus, water stored within a leaf could supplement that provided by the roots, thereby extending the duration of gas exchange activities for perhaps about two hours longer than otherwise, which could be critically important for a species when soil salinity constrains the supply of water to the leaves.

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725

Table 1. Abbreviations

Parameter	Symbol	Unit
Calculation with extracellular water included	+	
Calculation without extracellular water	-	
Water mass difference	Δw	g
Leaf water potential gradient	$\Delta\Psi$	MPa
Leaf dry mass	DM	g
Leaf fresh mass	FM	g
Maximum leaf fresh mass	FM_{max}	g
Water storage capacitance	$\mathbf{Q}$	g $MP^{-1}m^{-2}$
Water storage capacitance at domain 2	$\mathbf{Q_e}$	g $MP^{-1}m^{-2}$
General water storage capacitance	$\mathbf{Q_g}$	g $MP^{-1}m^{-2}$
Water storage capacitance at domain 1	$\mathbf{Q_x}$	g $MP^{-1}m^{-2}$
Relative water content	RWC	%
RWC at turgor loss point	RWC_{π}^{0}	%
RWC at full turgor	RWC_{π}^{100}	%
RWC at the transition between domains 1 and 2	RWC_x	%
Relative water deficit	RWD	%
RWD at turgor loss point	RWD_{π}^{0}	%
RWD during domain 2	RWD_e	%
RWD during domain 1	RWDx	%
Leaf area	S	cm^2, m^2
Maximum leaf water content	WC_{max}	g
Extracellular water content	WC_x	g
Pressure gradient	ΔP	MPa
RWC difference	ΔRWC	%
Fractional decrease in a cell's water volume	$\Delta V/V$	
Volumetric modulus of elasticity	$\boldsymbol{\varepsilon}$	MPa
Bulk modulus of elasticity at domain 2	$\boldsymbol{\varepsilon_e}$	MPa
General bulk modulus of elasticity	$\boldsymbol{\varepsilon_g}$	MPa
Bulk modulus of elasticity at domain 1	$\boldsymbol{\varepsilon_x}$	MPa
Leaf water potential	Ψ_{leaf}	MPa
Leaf water potential at the transition between domains 1 and 2	Ψ_x	MPa
Leaf water potential at full hydration	Ψ_{100}	MPa
Leaf water potential at turgor loss point	ψ_{π}^{0}	MPa
Osmotic potential at full turgor	ψ_{π}^{100}	MPa

Table 2. Summary of PV curve components and their calculations as previously described (Scholander *et al.*, 1964, Turner, 1988, Tyree & Ewers, 1991, Bartlett *et al.*, 2012).

Parameter	Symbol	Unit	Calculation
Water storage capacitance	**Q**	g MP^{-1} m^{-2}	$Q = \frac{\Delta w}{\Delta \Psi} \frac{1}{S}$ $= \frac{\Delta RWC . WC_{max}}{\Delta \Psi} \frac{1}{S}$
Bulk modulus of elasticity	**ε**	MPa	$\varepsilon = \frac{\Delta P}{\frac{\Delta V}{V}} = \frac{\Delta \Psi}{\Delta RWC}$
Maximum water content	$\mathbf{WC_{max}}$	g	$WC_{max} = FM_{max} - DM$
Relative water content	**RWC**	%	$RWC = (FM - DM)/WC_{max}$
Leaf water potential gradient	**ΔΨ**	MPa	$\Delta \Psi = \lvert \Psi_{tlp} - \Psi_{100} \rvert$
Relative water content deficit	**RWD**	%	RWD = 100 - RWC

Table 3. Properties of fully expanded, field-grown, sun leaves of *A. marina* used for measurements of PV curves and anatomical analyses. Values are mean ± se (n = 5, except for * where n = 15)

Parameter		**Symbol**	**Unit**	**Mean**	**se (n = 5)**
Leaf area		**S**	cm^2	14.8	1.1
Maximum fresh mass		**FM_{max}**	g	1.04	0.07
Dry mass		**DM**	g	0.38	0.02
Maximum water content		**WC_{max}**	g	0.66	0.05
Leaf mass per area		**LMA**	$g\ m^{-2}$	256.3	13.9
Maximum water content per dry mass		**$WC_{max}\ DM^{-1}$**	$g\ g^{-1}$	1.76	0.08
Leaf thickness			µm	588	10
Contribution to leaf thickness	Upper epidermis		%	3	0
	Hypodermis		%	36	1
	Palisade mesophyll		%	30	1
	Spongy mesophyll		%	13	0
	Trichome		%	19	1
Trichome	Density		cm^{-2}	218,758	8,783
	Volume*		$µm^3$	20,460	3,288 (n = 15)

Table 4. Leaf water relations of *A. marina* derived from three – domain PV curves as shown in Fig. 1. Values are mean ± se, n = 5. Note that symbols + or – denote whether extracellular water was included or excluded, respectively, in the calculations.

Parameter	Symbol	Unit	Mean	se (n = 5)
Relatively water deficit during domain 1	RWD_{x+}	%	13	1
Leaf water potential at RWC_x+	Ψ_x	MPa	-0.85	-0.03
Relative water deficit during domain 2	RWD_{e+}	%	12	1
Osmotic potential at full turgor	Ψ_π^{100}	MPa	-4.2	-0.1
Water potential at turgor loss point	Ψ_π^0	MPa	-5.1	-0.1
RWC at full turgor	RWC_π^{100}	%	87	1
RWC at turgor loss point, including extracellular water	RWC_π^{0+}	%	75	3
RWC at turgor loss point, excluding extracellular water	RWC_π^{0-}	%	86	2
General bulk modulus of elasticity	ε_g	MPa	21	2
Modulus of elasticity for domain 1	ε_e	MPa	7	1
Modulus of elasticity for domain 2	ε_x	MPa	37	4
General water capacitance	Q_g	g m^{-2} MPa^{-1}	22	2
Water storage capacitance for domain 1	Q_x	g m^{-2} MPa^{-1}	69	6
Water storage capacitance for domain 2	Q_e	g m^{-2} MPa^{-1}	13	2

Table 5. Composition of *A. marina* petiolar gel. Values are mean percent dry mass of the total dry mass of the polysaccharides named in the table below ± se, n = 3. Abbreviations: Rha: Rhamnose, Ara: Arabinose, Xyl: Xylose, Man: Mannose, ManUA: Mannuronic acid, Gal: Galactose, GalUA: Galacturonic acid, Glc: Glucose, GlcUA: Glucuronic acid. A: esterified uronic acid analysis, B: unesterified uronic acid analysis. NA: not applicable.

	Rha	**Ara**	**Xyl**	**Man**	**ManUA**	**Gal**	**GalUA**	**Glc**	**GlcUA**
A (%)	2.07	83.47	0.97	0.23	NA	10.60	NA	2.67	NA
se	0.19	0.32	0.09	0.03	NA	0.29	NA	0.22	NA
B (%)	2.23	83.17	0.80	0.23	0.00	10.53	0.00	0.53	2.50
se	0.19	0.50	0.06	0.03	NA	0.20	NA	0.15	0.15

Figure legends:

Fig. 1. A representative pressure volume (PV) curve of an *Avicennia marina* leaf grown at Batemans Bay, New South Wales, Australia: (a) Relative water content, calculated with total leaf water content, (RWC+, %) as a function of leaf water potential in absolute value ($|\Psi_{leaf}|$, MPa). Key points and their x, y coordinates are indicated by open symbols. In domain 1, ***A*** *(open circle)* shows the point of full hydration at which $|\Psi_{leaf}|$ was 0.1 MPa and leaf RWC was set at 100%; the transition between domains 1 and 2 is indicated by ***B*** *(open diamond)*; ***C*** *(open triangle)* is the turgor loss point at the transition between domains 2 and 3. (b) Inverse leaf water potential in absolute value ($|1/\Psi_{leaf}|$, MPa^{-1}) as a function of relative water deficit, calculated with total leaf water content, (RWD+, %). The full data set is plotted in the inset graph, whereas points representing extracellular water (points ***A*** to ***B*** in (a)) were excluded from the data set plotted in the main graph. The main graph shows relationships among calculated values of Ψ_{leaf} at the turgor loss point (point **c,** open circle), the y intercept ($\mathbf{d_0}$), and the intercept ($\mathbf{d_x}$) at RWD+ = RWD_x+, i.e. relative water deficit during domain 1. At point **c**, $|1/\Psi_\pi^0| = 0.2$ so $|\Psi_\pi^0| = 5$ MPa. The linear regression between $|1/\Psi_{leaf}|$ and RWD+ after the turgor loss point was reached followed the equation: $y = -0.0034x + 0.2682$ ($r^2 = 0.98$). Therefore, $|1/\Psi_{leaf}|$ at $\mathbf{d_0} = 0.2682$, and at $\mathbf{d_x} = 0.2376$ as RWD+ at $\mathbf{d_x} = 11$. Accordingly, $|\Psi_{leaf}|$ at $\mathbf{d_0} = 3.7$ MPa and at $\mathbf{d_x}$ (Ψ_π^{100}) = 4.3 MPa.

Fig. 2. Transverse sections of (a) petiole, (b) leaf midvein, and (c) leaf lamina of *A. marina*. Symbols: B: bundle sheath extension; C: cisternae; F: fimbrial vein; H: hypodermis; P: palisade mesophyll; S: spongy mesophyll; T: trichomes; V: vascular bundle. Yellow arrows indicate mucus secretion glands in (a) and salt secretion glands in (b) and (c). Black arrow indicates stomate. Bars are 100 µm.

Fig. 3. Trichomes from the abaxial surface of *A. marina* leaves at different hydration states viewed with (a) bright-field microscopy and (b-e) cryo-SEM. (a) Trichome general structure. Symbols: ca: cap cell, st: stalk cell, ba: basal cell. (b) Both cap and stalk cells were filled with water at $\Psi_{leaf} = -0.1$ MPa. (c, d) Cap cells were collapsed but stalk cells maintained their shapes and were filled with gas at $\Psi_{leaf} = -0.7$ MPa. (e) The lower stalk cell maintained its shape while the cap and upper stalk cell were shrivelled and collapsed at $\Psi_{leaf} = -4.3$ MPa. Bars are 20 µm. (f) Diagrammatic representation of changes in trichomes with dehydration, showing a trichome fully expanded and filled with water (blue) at $\Psi_{leaf} = -0.1$ MPa, shrunken, filled with gas (white) and with a collapsed cap cell at $\Psi_{leaf} = -0.25$ MPa, and shrunken, filled with gas, and with the cap and upper stalk cells collapsed at $\Psi_{leaf} = -4.3$ MPa.

Fig. 4. Detection of Ψ_{leaf} threshold for draining of water-filled trichomes during dehydration in *A. marina* leaves. (a) Trichome layer covering abaxial leaf surface viewed under white light showing the cap cells (grey) and underline stalk cells (circular back area) of the trichomes and (b) under blue light showing the absence of detectable auto-fluorescence which could interfere with identification of the green fluorescence emitted by fluorescein under blue-exciting light. (c, d and movie 1 (Supplement S2)) The spread of fluorescein over the wet cap surface of water – filled trichomes ($\Psi_{leaf} = -0.1$ MPa). The images were collected at 10s (c) and 2 min (d) after fluorescein was applied to the cap surface at a distance approximately 1 mm from the observed area. Fluorescence intensity was initially weak (c) and increased with time (d) as more fluorescein diffused across the observed area. (e-f and movie 2 (Supplement S3)) Uptake of fluorescein applied to the cap surface of trichomes when $\Psi_{leaf} = -0.25$ MPa. The observed area partly included the place where fluorescein was applied. Fluorescence from the dye drop was saturating (white area in e), and reflected by surrounding trichomes (green area in e). The drop of fluorescein initially maintained its shape on the cap surface and then rapidly disappeared a minute later when the drop was absorbed by underlying trichomes (f). Note that some stalk cells were filled with dye (arrow in f) while in others, dye remained in the wall of cap and stalk cells and the surrounding area appeared black. Bars are 0.05 mm.

Fig. 5. Petiolar cisternae in leaves of *A. marina*. Distribution of cisternae (✱✱) in (a) transverse and (b) longitudinal sections as visualized by bright- field microscopy. Cryo-SEM micrographs of cisternae

filled with water when Ψ_{leaf} = -0.1 MPa (c), or air and droplets when Ψ_{leaf} = -0.1 MPa (c, d), Ψ_{leaf} = -0.7 MPa (e) and Ψ_{leaf} = -6 MPa (f). Bars are 50 µm in (a, b), and 25 µm in (c-f).

Fig. 6. Cryo-SEM micrographs of transverse sections through (a, b) pith parenchyma in the midvein and (c, d) paradermal sections through spongy mesophyll cells in the lamina of leaves of *A. marina* differing in leaf water status. Arrows point at extracellular spaces filled with ice, indicating the presence of liquid water (a, c) at Ψ_{leaf} = -0.06 MPa and with gas (b,d) at Ψ_{leaf} = -1 MPa. Bars equal 20 µm in (a, b) and 10 µm in (c, d).

Fig. 7. Plasmolysis in different cell types of leaves of *A. marina* after turgor had been lost ($\Psi_{leaf} \approx$ -6 MPa). (a) Petiolar collenchyma, (b) petiolar parenchyma surrounding gas-filled cisternae, (c) hypodermis, and (d) palisade mesophyll. Arrows indicate gaps between cell walls and cell membranes. Bars are 25 µm in (a, b), and 20 µm in (c, d).

Fig. 8. Diagrammatic summary of changes in the leaf lamina during dehydration through domains 1, 2, and 3 of the PV curve shown in Fig. 2. The major tissue layers of hypodermis (H), palisade (P), spongy mesophyll (S), and trichomes (T) are indicated as in Fig. 3. Salt secretion glands (purple) occurred on both leaf surfaces while stomata (pink dots) occurred only on the abaxial surface. At maximum hydration in Domain 1, all cells were turgid. Extracellular water (blue) occurred in a few extracellular spaces of the mesophyll where cells were in close apposition as in Fig. 6c. Trichomes were filled with water as in Fig. 4b, f. At the beginning of Domain 2, extracellular water was absent from spongy mesophyll as in Fig. 6d and trichomes as in Fig. 4c, d, f. Finally, after turgor was lost at -5 MPa in Domain 3, plasmolysis was evident with further dehydration to -6 MPa in most cell types, including hypodermis (Fig. 7c), palisade (Fig. 7d) and spongy mesophyll cells (data not shown). Plasmolysed cells are indicated by thin white gaps between cell walls and cell membranes. While the leaves declined in thickness during dehydration, cellular connections were maintained with no sign of cell collapse, except in the shrivelled, hollow cap and stalk cells of trichomes (Fig. 4e, f). The variation in panel size and colours indicate progressive shrinkage of the leaf lamina during dehydration from Domain 1 through Domain 3.

Supplement S1. Reanalysis of the data set from Fig.1, assuming domain 1 represented a "plateau effect". (a) Relative water content, calculated without extracellular water, (RWC-, %) as a function of leaf water potential in absolute value ($|\Psi_{leaf}|$, MPa) with the fully hydrated status (100% RWC) set at ***B** (open diamond)***.** Open dots indicate extracellular water (RWD_x-). (b) Inverse leaf water potential in absolute value ($|1/\Psi_{leaf}|$, MPa^{-1}) as a function of relative water deficit, calculated without extracellular water (RWD-,%). For reference, the full data set in Fig.1b, including the extracellular water, is plotted in the inset graph. Point **c (**open circle**)** is the turgor loss point and $|\Psi_\pi^0|$ = 5 MPa as in Fig. 1b. With the changes excluding extracellular water in RWD calculation, the equation for the linear regression between $|1/\Psi_{leaf}|$ and RWD- after **c** was reached was $y = -0.003x + 0.2313$ ($r^2 = 0.98$). Thus, the y intercept at point **d,** $|1/\Psi_\pi^{100}| = 0.2314$ so $|\Psi_\pi^{100}| = 4.32$ MPa.

Supplement S2: Movie showing diffusion of fluorescein across an abaxial leaf surface when Ψ_{leaf} = -0.1 MPa and trichomes were full of water (see Fig. 4 for further details).

Supplement S3: Movie showing absorption of fluorescein by draining trichomes when Ψ_{leaf} = - 0.25 MPa (see Fig. 4 for further details).